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(54) Title: METHODS FOR ISOLATING HERPES VIRUS THYMIDINE KINASE-ENCODING DNA		
(57) Abstract Methods for isolating thymidine kinase-encoding DNA of a herpes virus are described. These methods utilize degenerate primers based on regions of relatively conserved amino acid sequence in herpes virus thymidine kinase proteins to initiate a polymerase chain reaction which yields large amounts of the thymidine kinase-encoding DNA. The methods are illustrated in the isolation of the thymidine kinase gene of feline herpes virus, which can be used to construct recombinant thymidine kinase-negative feline herpes viruses for purposes of constructing live vaccines and expression vectors. In addition, the regulatory elements of the feline herpes virus thymidine kinase gene are useful in the construction of recombinant DNA vectors.		

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METHODS FOR ISOLATING HERPES VIRUS THYMIDINE KINASE-
ENCODING DNA

SUMMARY OF THE INVENTION

In one aspect, the present invention provides methods for
5 isolating and cloning thymidine kinase-encoding DNA from members of
the herpesvirus family. These methods comprise mixing thymidine
kinase-encoding DNA of a herpes virus with a mixture of primers, said
mixture of primers containing primers that encode, in all variations
possible due to the degeneracy of the genetic code, all variations of
10 a first sequence of relatively conserved amino acids of herpes virus
thymidine kinase proteins, and primers that encode, in all variations
possible due to the degeneracy of the genetic code, sequences
complementary to sequences that encode all variations of a second
sequence of relatively conserved amino acids of herpes thymidine
15 kinase protein; amplifying the thymidine kinase-encoding DNA by the
polymerase chain reaction; and isolating the amplified DNA. The
invention also provides mixtures of primers useful in practicing the
method.

In a second aspect, the invention provides DNA compounds that
20 encode the thymidine kinase (TK) of feline herpes virus (FHV), also
known as feline viral rhinotracheitis virus. These DNA compounds,
which include recombinant DNA vectors that comprise TK-encoding DNA,
are particularly useful in generating recombinant FHVs that can be
used as vaccines and expression vectors. As used herein, "TK-
25 encoding DNA" refers to DNA that encodes a portion of the amino acid
residue sequence of a herpes thymidine kinase and can include
sequences that flank such DNA on a herpes virus genome.

In a third aspect, the invention provides methods for
constructing thymidine kinase negative FHVs. These methods comprise
30 constructing a recombinant DNA vector that encodes a non-functional
FHV TK gene; transfecting an FHV-permissive host cell with a mixture
of TK-positive FHV and the plasmid that encodes a non-functional TK
gene; isolating the progeny virus; infecting an FHV-permissive host
cell with said progeny virus to produce thymidine kinase-negative
35 virus; and isolating said replicating TK-negative virus. The virus
can be isolated by methods well known in the art, for example, by
culturing the infected host cells under conditions where only
thymidine kinase negative virus replicates. A portion of these TK-

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minus viruses will contain the plasmid TK-minus-encoding DNA recombined into the viral genome. The recombinant FHV's produced by the method are also an important aspect of the present invention.

Thymidine kinase negative herpesviruses can also be isolated by drug selection, e.g., 100 μ g/ml thymidine or arabinoside in the culture medium. These mutations can be spontaneous or mutagen induced. Preferably these mutations involve deletions and are made via recombinant DNA.

Thus, in a fourth aspect, the present invention provides FHV's that do not contain a functional TK gene, and so are referred to as "TK-negative" and "TK-minus" FHV's. Such TK-minus FHV's are useful as vaccines, because in comparison to wild-type FHV, a TK-minus FHV is attenuated in its ability to render an infected animal ill yet can still elicit an immune response that protects against further infections by wild-type FHV. In addition, the invention provides recombinant TK-negative FHV's that comprise an expression cassette inserted into the TK gene sequence of the FHV. Such an insertion renders the FHV TK-negative but, more importantly, also renders the FHV into an expression vector. As used herein, "expression cassette" refers to a recombinant DNA sequence that encodes a promoter operably linked to a coding sequence, such that when the expression cassette is present in an FHV-infected host cell, the promoter can drive transcription of an mRNA (e.g., that encoding feline leukemia virus (FeLV) envelope protein) that is capable of being translated into protein by the cell.

Figure 1: Restriction Endonuclease Analysis of Recombinant FHV.

(A) EcoRI restriction endonuclease digestion of parental FHV UT88-1729 (1) and recombinant araT-resistant FHV-113 (2).

(B) The EcoRI-digested DNA visualized in panel A was transferred to nitrocellulose and tk sequences were identified by using a nick-translated hybridization probe comprising the 3' portion of the FHV tk gene. The tk-containing 6.6 kb EcoRI fragment in the parental virus(1) is reduced by approximately 345 bp in the deletion-containing virus FHV-113 (2). Molecular size markers (HindIII- λ DNA) are indicated. Several other EcoRI fragments which also do not co-migrate in both viral DNAs map to the repeat regions of FHV (44); variation in the molecular size of these regions has been noted in unrelated experiments in which FHV is plaque-purified from a

population.

Figure 2: Results of Assay of tk Enzymatic Activity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for isolating any
5 thymidine kinase-encoding DNA of any herpes virus. These methods
comprise (a) mixing herpes virus DNA with a mixture of primers, said
mixture of primers containing primers that encode, in all variations
possible due to the degeneracy of the genetic code, all variations of
a first sequence of relatively conserved amino acids of herpes virus
10 thymidine kinase proteins, and primers that encode, in all variations
possible due to the degeneracy of the genetic code, sequences
complementary to sequences that encode all variations of a second
sequence of relatively conserved amino acids of herpes thymidine
kinase protein; (b) amplifying the thymidine kinase-encoding DNA by
15 the polymerase chain reaction; and (c) isolating the amplified DNA.

The first step in these methods involves mixing herpes virus DNA
with a mixture of primers. "Primers," as used herein, refers to
oligonucleotides that can be extended by the action of a DNA
polymerase in the polymerase chain reaction. The design of the
20 primers useful in the methods of the present invention is dictated by
two factors: (i) the primers must be amenable to use in the
polymerase chain reaction; and (ii) the primers must be able to
hybridize to a single-stranded DNA that either encodes thymidine
kinase or is the complement to a single-stranded DNA that encodes
25 thymidine kinase. Each of these factors is discussed in detail
below.

The polymerase chain reaction is a well known technique,
described in U.S. Patent 4,683,202 and other applications and patents
for amplifying DNA. Primers for use in the polymerase chain reaction
30 are designed to be able to hybridize with at least one strand of the
double-stranded target DNA sequence to be amplified. Briefly stated,
the polymerase chain reaction involves the following steps. First,
the double-stranded target sequence is denatured. Second, a first
primer is annealed to one strand of the denatured target DNA while a
35 second primer is annealed to the other strand of the denatured target
DNA. The two primers anneal to the target DNA at sequences removed
from one another and in orientations such that the extension product
of one primer, when separated from its complement, can hybridize to

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the other primer. Once a given primer hybridizes to the target sequence, the primer is extended by the action of DNA polymerase. The extension product is then denatured from the target sequence, and the process is repeated.

5 In successive cycles of this process, the extension products produced in earlier cycles also serve as sites for DNA synthesis. Beginning in the second cycle, the product of amplification begins to accumulate at a logarithmic rate. This product is a double-stranded DNA molecule, one strand of which contains the sequence of the first
10 primer, which is followed by the sequence of one strand of the target DNA, which, in turn, is followed by a sequence complementary to the sequence of the second primer. The other strand of the product is complementary to the first strand just described.

Several aspects of the polymerase chain reaction are important
15 to note for purposes of the present invention. First, primers can be designed with convenient restriction enzyme recognition sequences located at or near the 5' end of the primer. In the formation of extension products in the polymerase chain reaction, new nucleotides are added beginning at the 3' end of the primer. These new
20 nucleotides are added only if the 3' end of the primer is hydrogen-bonded to the target sequence, so the sequences that encode the restriction enzyme recognition sequence must be located at or near the 5' end of the primer. For example, one primer might contain a BamHI restriction enzyme recognition sequence at its 5' end, while
25 the other primer might contain an EcoRI restriction enzyme recognition sequence at its 5' end. After amplification, the product would be digested with BamHI and EcoRI restriction enzymes and cloned into an appropriately cleaved cloning vector. The presence of such restriction enzyme recognition sites in the product greatly
30 facilitates cloning.

Second, the target of amplification can be single-stranded DNA. Although the polymerase chain reaction procedure described above involved the assumption that the target was double-stranded, a single-stranded target sequence can serve as well in the
35 amplification process. After the first cycle of amplification of a single-stranded target, the reaction mixture essentially contains a double-stranded target molecule consisting of the single-stranded target and its complementary strand, so successive cycles of

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amplification proceed as described above.

The second important factor in designing the mixture of primers used in the methods of the present invention is that the primers must be able to hybridize to DNA that encodes the thymidine kinase of any herpes virus. The thymidine kinase genes of known herpes viruses are quite diverged and contain only very short and interspersed regions of amino acid identity (see Kit, 1985, Microbiol. Sciences 2:369-375). For instance, pairwise comparison of the HSV1 TK (McKnight, 1980, Nuc. Acid Res. 8:5949) protein with that of pseudorabies virus (PrV, see U.S. Patent No. 4,514,497) or varicella zoster virus (VZV, see Davison et al., 1986, J. Gen. Virol. 67:1759-1816) reveals only 7 colinear regions in which all 4 amino acids within any stretch of 4 amino acids are identical between pairs. The points of identity are not necessarily conserved between pairwise comparisons. Additional herpes virus TK genes that further illustrate this divergence include HSV-2 (Swain et al., 1983, J. Virol. 46:1045-1050), MaHV (Otsuka et al., 1984, Virol. 135:316-330), and IBR (EPO 226,029). Efforts to isolate the FHV TK gene via standard hybridization methods using the known TK genes as probes were not fruitful, because the divergence of TK proteins and the degeneracy of the genetic code renders such hybridization techniques too nonspecific.

The methods of the present invention, however, provide a way to isolate and clone any herpes thymidine kinase-encoding DNA. These methods utilize oligonucleotide primers that encode, in all variations possible due to the degeneracy of the genetic code, all variations of the very small regions of amino acid sequence homology between known herpes virus thymidine kinases. Although the number of primers in an amplification reaction designed to isolate TK-encoding DNA is quite large, only those primers that hybridize are amplified in the reaction. Thus, the methods of the present invention are quite specific in that the use of short, highly-degenerate oligonucleotides that encode (or are complementary to DNA that encodes) short, moderately conserved amino acid sequences requires that (i) two primers anneal; (ii) to opposite strands; and (iii) yield a product of the size expected. The methods of the invention are illustrated by the isolation of the feline rhinotracheitis virus TK gene. This virus, referred to herein as feline herpes virus (FHV)

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contains a TK gene never before isolated or characterized. The FHV TK gene was obtained using short, highly-degenerate oligonucleotide primers by the methods of the present invention.

Although the present invention is not limited to particular
5 primers, because other regions of conserved amino acid sequence than those exemplified herein exist, the invention does provide preferred primers for use in the method for isolating TK-encoding DNA. Because these primers can contain non-homologous DNA at the 5' end of the primer (i.e., restriction enzyme recognition site-encoding DNA, as
10 described above), and because the primers encode a relatively conserved amino acid sequence, the preferred primers of the invention are defined as comprising a coding sequence (or the complement thereof) for a relatively conserved amino acid sequence.

It should be noted that a given "conserved amino acid sequence"
15 can consist of two or more sequences, and thus the methods of the invention refer to "all variations of a conserved amino acid sequence." For example, residues 55-60 (numbering of amino acid residues for purposes of designating conserved regions refers to the HSV 1 TK amino acid sequence) are relatively conserved within the
20 herpes virus thymidine kinases, i.e., this region is not identical in every herpes virus thymidine kinase at each position in the sequence but is still recognizable as a region of homology. In constructing primers for such a conserved region for purposes of the present invention, however, one need only design the primers to encode, in
25 every variation possible due to the degeneracy of the genetic code, each variation of the conserved sequence. The preferred primers of the invention are depicted in Table 1, below, by reference to the conserved amino acid sequences, some of which are variant as just described. The amino acid sequences are given in one-letter code,
30 described in Table 2. The position of the amino terminal residue in the conserved sequence (relative to the HSV-1 TK) is indicated on the first line of Table 1. The virus in which a particular conserved sequence is found is indicated at the left side of each line. Because of the degeneracy of the genetic code, and because the method
35 of the invention utilizes primers that encode all possible coding sequences for a conserved amino acid sequence (and the complementary strands of such coding sequences), the actual number of primers used in the method that encode a given conserved amino acid sequence is

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indicated in the line entitled "primer degeneracy." Finally, Table 1 also depicts the actual length of the portion of the primer that encodes the conserved sequence. In some cases, due to the variability of the nucleotide in the third position of a codon, this length can be one nucleotide shorter than the calculated three nucleotides per conserved amino acid.

Table 1

Primers for Isolating TK-encoding DNA

10		55	61	164	220	287
	HSV-1	DGPHG	GKTT	DRHP	RPGE	DTLF
	VZV	DGAYG	GKTT	DRHP	RPGE	DTLF
	PrV	DGAYG	GKST	DRHP	RAGE	DTLF
	MaHV	DGPHG	GKST	DRHA	RPGE	----
15	Primer					
	length	14	11	11	11	12
	Primer					
	degeneracy	256	112	48	196	96

Table 2

Amino Acid Abbreviations

	Amino acid	Three-letter abbreviation	One-letter abbreviation
25	Alanine	Ala	A
	Arginine	Arg	R
	Asparagine	Asn	N
	Aspartic acid	Asp	D
	Cysteine	Cys	C
30	Glutamine	Gln	Q
	Glutamic acid	Glu	E
	Glycine	Gly	G
	Histidine	His	H
	Isoleucine	Ile	I
35	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
	Phenylalanine	Phe	F

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	Proline	Pro	P
	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
5	Tyrosine	Tyr	Y
	Valine	Val	V

The primers shown in Table 1 are used in pair wise combinations. For example, one could isolate a TK-encoding DNA by amplifying with a primer pair in which the first primer encodes the conserved amino acid sequence beginning at position 55 (as shown in Table 1, this first primer would be a mixture of primers that comprise sequences that encode DPGHG and sequences that encode DGAYG in all variations possible due to the degeneracy of the genetic code) and in which the second primer comprises sequences that are complementary to sequences that encode the conserved amino acid sequence beginning at position 220 (as shown in Table 1, this second primer is a mixture of primers that comprise sequences complementary to sequences that encode RPGE and sequences that encode RAGE in all variations possible due to the degeneracy of the genetic code). The expected size of the product of amplification would be about 495 base pairs (bp) in length ($220 - 55 - 165$, $165 \times 3 = 495$). The amplification reactions can be carried out on a Perkin-Elmer Cetus Instruments Thermal Cycler using the thermostable DNA polymerase of Thermus aquaticus in accordance with the manufacturer's protocol. In addition, reaction protocols for the polymerase chain reaction using a thermostable polymerase are described U.S. Patents 4,683,195 and 4,683,202 and U.S. patent applications S.Nos. 063,647 (filed June 17, 1987) and 899,513 (filed August 22, 1986), all of which are incorporated herein by reference. In addition, methods for amplifying DNA using the DNA polymerase of T. aquaticus are described in Saiki et al., 1988, Science 239:487-491.

To isolate the FHV TK-encoding DNA of the present invention, primer pairs as shown in Table 1 and including 5' extensions containing restriction endonuclease recognition sites (to facilitate subsequent cloning of the product of amplification) were used in the method of the present invention. The amplification reactions were carried out on a Perkin-Elmer Cetus Instruments Thermal Cycler using

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the thermostable DNA polymerase of Thermus aquaticus in substantial accordance with the manufacturer's protocol, except as follows. From 30 ng to 1 μ g of FHV DNA (UC-D strain of FHV, obtained from Niels Pedersen, University of California Veterinary School, Davis, Ca) and
5 100 to 800 pmol of degenerate primers were used in each 50 μ l reaction. The thermal cycling included an initial 5 cycles with an annealing step at 37°C.

One pairwise combination of primers yielded the expected product. This primer pair was designed to amplify the region between
10 and containing the conserved sequences beginning at position 55 and 164 as depicted in Table 1. Thus, the primer pairs had the following sequences. The first primer was:

5'-TCAAAGCTTGAYGGNSCNAYGG-3'

The second primer contained equal parts of the following primers:

15 5'-CTCGAATTCGSRTGNCGRTC-3' and

5'-CTCGAATTCGSRTGYCTRTC-3',

In the sequence of the primers shown above, A is a deoxyadenine residue, T is a thymidine residue, C is a deoxycytidine residue, G is a deoxyguanine residue, N represents that the primer is a mixture of
20 primers in which each of the four nucleotides can occur at the position indicated, R represents that the primer is a mixture of primers in which either of the two purine nucleotides (G and A) can occur at the position indicated, Y represents that the primer is a mixture of primers in which either of the two pyrimidine nucleotides
25 (C and T) can occur at the position indicated, S represents that the primer is a mixture of primers in which either a G or C nucleotide can occur at the position indicated. Those skilled in the art will note that the first primer encodes a HindIII restriction enzyme recognition sequence (5'-AAGCTT-3') near the 5' end and that the
30 second primer encodes an EcoRI restriction site (5'-GAATTC-3') at the 5' end.

The expected product from the primer pair described above had a length of about 350 bp (164 - 55 = 109, 109 x 3 = 327), and after the amplification reaction mixture was digested with restriction enzymes
35 EcoRI and HindIII, the reaction mixture was loaded onto an acrylamide gel and subjected to electrophoresis. The approximately 350 bp product was excised from the gel and ligated with EcoRI-HindIII-digested BlueScript plasmid vectors (BlueScript is a tradename of

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Stratagene Corporation, 3770 Tansey Street, San Diego, CA 92121, and the vectors were used in substantial accordance with the manufacturer's protocol). The recombinant plasmids were sequenced to confirm that the approximately 350 bp EcoRI-HindIII restriction fragment encoded FHV thymidine kinase. This determination was made by comparing the amino acid sequence encoded by the coding sequence to that of other known herpes virus thymidine kinase proteins, which, although too divergent for cloning by hybridization are similar enough that such a determination is practicable. The approximately 350 bp fragment was then used to isolate the entire FHV TK gene from genomic libraries of FHV by labeling the 350 bp fragment, contacting the labeled fragment with the library under hybridizing conditions, and isolating the clones in the library that hybridized to the fragment. In this manner, the entire TK gene was isolated on two plasmids. The first, designated pTK3.8 is a 3.8 kb SalI-HindIII restriction fragment of FHV strain UC-D cloned into a Bluescript vector (purchased from Stratagene, La Jolla, Ca), and the second, designated pTK5.4deltaBam is a 1.7 kb HindIII-BamHI restriction fragment of FHV strain UC-D cloned into a Bluescript vector.

The DNA sequence of the FHV TK gene was determined and is set forth below. Those skilled in the art recognize that there can be difficulty in interpreting DNA sequencing gels and that the sequence depicted below may differ from the actual sequence in a few nucleotide positions. However, by using the methods of the invention, one can isolate any herpes virus TK-encoding DNA sequence. In addition, the present invention allows the position of the TK gene to be identified to a particular restriction fragment of the feline herpes virus genome. Rota *et al.*, 1986, Virology 154:168-179, reported a restriction map of a feline herpes virus that contains a SalI restriction fragment about 20 kilobases (kb) in length. This restriction fragment, termed the Sal A fragment, contains the feline herpes virus TK gene. Most feline herpes viruses are substantially homologous to this reported virus, which enables one of skill in the art to isolate the TK-encoding DNA compounds of the present invention merely by cloning the appropriate restriction fragment. The sequence is numbered to facilitate description of the sequence; the numbers appear at the left-hand side of the sequence. Only the coding strand of the sequence is depicted. Underlined portions of the sequence are

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described on the line above. "N" represents that the nucleotide in the designated position might be either A, G, T, or C.

Nucleotide Sequence of the Thymidine Kinase Gene of Feline Herpes Virus, Strain UC-D

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5   1 5'-GTATAACCAC AGATCTGTAT GTTCAACCTC ACGACGTTGA TGTCTTACTA
    51   GTGTATCCAT ATTTTGAAAA CGACACGTTT TCAGCTCAAT TAGAAAACAT
   101   ATACCACCCC CTTCTCCCTC AAATTGTATA GTACATACAC AATCAGTCGG
   151   CGACGACCGA AGTTAACCTC ACATGCTAGG TACACGCCCT TAGCCTTTTT

                                CAAT box
  10  201   AAGAGACTCT GCGGATACAG AGCCGCCCAA TAAACACTCG AGTCGGTCGG

                                TATA box
    251   TATATACTCC ACTCGCAGAG GTCGAGGATA TATCGCGCTT GAGGACAGCA
    301   TAAAAGCGAT TGTGGNATCG AATTCCAGCC CGGAGCCTCA ATCCGACACT

                                start of coding sequence
  15  351   GCGTCGTTGT TCACGTTTCA TCATACACAG ATCAGACGAT GCGGAGTGGA
    401   ACCATCCCCG TTCAGAATGA AGAGATTATT AAATCACAGG TGAATACTGT
    451   CCGCATTTAC ATAGATGGTG CCTATGGAAT AGGTAAGAGT TTAACGGCGA
    501   AGTACCTGGT CAGAGCGGAT GAAAATCGAC CGGGATATAC TTACTACTTC
    551   CCAGAACCAA TGCTATACTG GCGTAGTCTC TTTGAAACTG ATGTTGTCCG
  20  601   TGGTATCTAT GCCGTCCAGG ACCGGAAACG ACGTGGTGAA TTATCAGCTG
    651   AAGATGCTGC CTATATCACC GCCCACTATC AAGCAAGATT TGCCGCACCA
    701   TACCTTCTTT TACATTCCAG ACTATCCACA ATAACAGGAT ATCAGAAAGT
    751   TGTATGTGAG GAACACCCCG ACGTGACCCT AATCATAGAT AGACACCCTC
    801   TCGCCTCTCT GGTCTGTTTC CCACTCGCAA GATATTTTGT GGGTGATATG
  25  851   ACTCTTGGGT CTGTACTTAG TCTAATGGCA ACACTTCCAC GAGAACCCTC
    901   TGGTGAAAT CTAGTTGTAA CAACCTTGAA TATCGAGGAA CATTGGAAGC
    951   GTCTCAGGGG ACGCTCAAGA ACCGGAGAAC AGATAGACAT GAAGCTAATT
   1001   CACGCACTAC GCAATGTATA TATGATGTTG GTACATACTA AGAAATTTTT
   1051   AACAAAAAAT ACTAGTTGGC GTGATGGGTG GGGGAAGCTT AAAATTTTCT
  30  1101   CCCACTATGA ACGGAATAGG CTGGTGAAA CTACAATAGT TTCCGATTCTG
   1151   ACGGAGTCAG ATTTATGTGA CACATTATTC AGTGTTTTCA AAGCCCGGGA
   1201   GCTCTCCGAC CAAAATGGAG ATCTACTTGA CATGCATGCA TGGGTCCTCG
   1251   ATGGACTTAT GGAAACCCTC CAAAATTTAC AGATCTTTAC TTTAAATCTG
   1301   GAAGGAACCC CTGATGAATG TGCCGCCGCC TTGGGAGCAC TGAGACAAGA
  35  1351   TATGGATATG ACATTTATAG CCGCATGTGA TATGCACCGT ATAAGTGAAG

                                end of coding sequence
   1401   CCTTGACGAT ATACCATTAA ACATTAGTGG TGTTCCTAT TACCCCCCTG
   1451   TGGTGAATGT GTGGAGGTCA GGGGATAATT GTATAATGAC CATCGTTTCA

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poly A

1501 TGAATAAAAT AACCGTGTGT GATGTGGATG TATTGATTAA TTGAATTTCT
 1551 CTTCGGGTTT TAGATCTTTA TAAGCGTAAA ACTGGTGTTC TAAATCCAAG
 1601 AGCCGGGTTT TTTGGAGGTT GGTACATCA TCGCCACAGC CCGTGGATTC
 5 1651 AAGCAATCTT ATGATGTGTT TGATAATATA CCTATCGATA TTCCTGATCA
 1701 TTGTATCGAG GATGTTGACT GGTACCGAT GATGGATAGA CCTGATGAGG
 1751 TGGCTGG-3'

The TK-encoding DNA sequence shown above, although isolated by the method of the present invention, can be constructed by synthetic means, i.e., by use of an automated DNA synthesizer, well known in the art. This TK-encoding DNA sequence is an important aspect of the present invention, as well as recombinant DNA vectors that comprise the sequence. In addition, the TK gene sequence shown above will be homologous to other TK-encoding DNA sequences of other FHV, if other FHV with a different TK-encoding DNA sequence exist. There may be a number of different FHV strains that may differ from one another only in a minor way. These FHV variants may encode thymidine kinase proteins that differ from the FHV TK protein encoded by the TK gene of the present invention in some way; however, such variations will occur in less than 10% of the amino acid residue positions. Such variant genes can be readily located by a variety of methods, including hybridization with the TK-encoding DNA of the present invention and comparison of genetic maps to locate analogous TK-encoding regions. Thus, the present invention provides TK-encoding DNA from any FHV.

The TK gene sequence depicted above contains a promoter (a sequence that includes a CAAT region and TATA box, which can include sequences upstream of the CAAT region and downstream to about the start of the coding sequence), coding sequence, and poly A signal. The promoter and poly A signal are important regulatory elements that, through the use of recombinant DNA technology, can be used to construct recombinant genes that drive expression of any desired gene product. Thus, the promoter and poly A portions of the FHV TK gene are also important aspects of the present invention.

The coding sequence of the TK gene encodes a thymidine kinase with the following amino acid residue sequence shown below. The sequence is listed from amino to carboxy terminus.

1 MASGTIPVQN EEIKSQVNT VRIYIDGAYG IGKSLTAKYL VRADENRPGY

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51 TYYFPEPMY WRSLFETDVV GGIYAVQDRK RRGELSAEDA AYITAHYQAR
 101 FAAPYLLLHS RLSTITGYQK VVCEEHPDVT LIIDRHPLAS LVCFFPLARYF
 151 VGDMTLGSLV SLMATLPREP PGGNLVVTTL NIEEHLKRLR GRSRTGEQID
 201 MKLIHALRNV YMMLVHTKKF LTKNTSWRDG WGKLKIFSHY ERNRLVETTI
 5 251 VSDSTESDLC DTLFSVFKAR ELSQNGDLL DMHAWVLDGL METLQNLQIF
 301 TLNLEGPDE CAAALGALRQ DMDMTFIAAC DMHRISEALT IYH

Those skilled in the art recognize that due to the degeneracy of the genetic code, a very large number of DNA sequences can be constructed that encode the thymidine kinase of the structure shown above. These
 10 DNA sequences are equivalent to the FHV TK-encoding DNA of the present invention.

The FHV TK gene is useful in the construction of infectious TK-minus FHV for use as attenuated FHV (feline rhinotracheitis virus) vaccines. The invention provides methods for constructing
 15 recombinant FHV's that comprise constructing a recombinant DNA vector that encodes a non-functional FHV TK gene; transfecting an FHV-permissive host cell with a mixture of TK-positive FHV and the plasmid that encodes a non-functional TK gene; isolating the progeny virus; infecting a feline herpes virus-permissive host cell with said
 20 progeny virus to produce thymidine kinase-negative virus; and isolating said replicating TK-negative virus. The resulting TK-negative virus will contain a mixture of virus, only a portion of which are rendered TK-negative as a result of recombination with the plasmid-borne TK sequences. The remaining portion of TK-minus
 25 viruses is a result of spontaneous mutation to the TK-minus state. The recombinant FHV's produced by the method are also an important aspect of the present invention.

A number of TK-minus herpes viruses are known that show reduced virulence and so can be used as attenuated virus vaccines (see, e.g.,
 30 European Patent Publication 226 029; and U.S. Patent 4,703,011 which describe bovine herpesviruses type 1 which fail to produce any functional thymidine kinase as a result of a deletion in the thymidine kinase gene. Also this patent refers to other herpes viruses). Before the present invention it was not known whether it was possible
 35 to make a viable thymidine kinase negative feline herpesvirus, how to make such a virus, whether such a virus would be virulent or avirulent in cats, or whether such a virus would produce a protective immune response in cats. The fact that tk negative pseudorabies

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virus is virulent in cats teaches against the idea that tk negative herpesviruses are avirulent in cats. It is only as a result of the instant invention that it has in fact been demonstrated that tk minus FHV's are avirulent and are protective vaccines. To obtain such an

5 FHV TK-minus vaccine to immunize cats and other susceptible animals against infection by wild-type FHV, one need construct a recombinant vector that encodes a non-functional FHV TK-gene but retains sufficient homology to the wild-type FHV TK gene and flanking sequences for recombination. Then, the non-functional TK gene is

10 recombined with TK-positive FHV DNA and TK-negative recombinants are selected. It is important to note that a "non-functional" TK gene includes a segment of FHV genomic DNA that is colinear with the FHV genome except for a deletion or insertion (e.g., insertion of an expression cassette, as described below) or point mutation that

15 renders the TK gene inoperative.

In a variation on this theme, the non-functional TK gene is modified to also include an expression cassette. In the preferred embodiment, this expression cassette will drive expression, when present in the animal immunized with the recombinant virus, of a

20 protein that will induce immunity to other infectious agents. For instance, feline leukemia virus (FeLV) is an infectious agent for which immunizing vaccines are needed. The FHV TK-minus recombinant viruses of the present invention are readily modified to encode FeLV proteins, such as the envelope, pol, or gag proteins, that, when

25 expressed in the immunized animal, will render the animal resistant to FeLV. Of course, the infectious recombinant FHV's of the present invention can express other genes for use in cats, cat cells, or other cells or animals susceptible to FHV infection. For example, the recombinant TK-minus FHV's can be used as vaccines against feline

30 infectious peritonitis (FIP) virus, calicivirus, rabies virus, feline immunodeficiency virus (FIV), feline parvovirus (panleukopenia virus), and feline Chlamydia; and as generalized expression vectors, i.e., to correct genetic defects or to provide additional growth,

merely by choice of the appropriate expression cassette.

35 To obtain recombination and insertion of foreign sequences within the FHV genome, it is necessary to flank the inserted sequence with FHV sequences; in the case of targetted insertion within the FHV TK gene, it is necessary to insert the foreign gene or expression

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cassette into the FHV TK gene and to flank this insert with (50 -5000 bp) colinear sequence including and/or surrounding the FHV TK gene. Insertion of an expression cassette within the FHV TK gene will generate a recombinant TK-minus FHV suitable as an attenuated live FHV vaccine.

The plasmids described below contain expression cassettes that can be inserted into the TK gene of the present invention; the resulting construct can be recombined with FHV to yield an FHV TK-minus recombinant virus illustrative of the invention. These expression cassettes demonstrate the ability of a variety of herpes virus promoters to drive expression of any protein, as illustrated by a beta-galactosidase marker protein, which is easily detected by chromogenic assays, in FHV-infected cells. Such promoters include the herpes simplex alpha-4 promoter ($\alpha 4$), which can be isolated from plasmid pRB403, described by Roizman *et al.*, 1982, *Proc. Natl. Acad. Sci.* 79:4917-4921, on a PvuII-BamHI restriction fragment; the cytomegalovirus immediate early promoter (CMVIE), which can be isolated from plasmid pCMV5027, described by Schaffner *et al.*, 1985, *Cell* 41:521-530, on a Sal I-SacII restriction fragment with minor repair (the Sal I site is from vector polylinker in pCMV5027, and is where a Pst I site exists upstream from the CMV promoter); and the FHV TK promoter described above, which can be isolated on a Sal I-EcoRI restriction fragment (the EcoRI site is about 100 bp upstream of the ATG that starts the coding sequence).

Each of these promoters were cloned into appropriate sites in the beta-galactosidase-encoding but promoter-less plasmid pON1, described by Spaete *et al.*, 1985, *J. Virol.* 56:135-143. Transfection and assay for beta-galactosidase expression in FHV-infected CRFK cells (available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852-1776, under the accession number ATCC CCL 94) or HSV1-infected Vero cells from the ATCC were essentially as described by Spaete *et al.*, 1985, *J. Virol.* 56:135-143, incorporated herein by reference. The results are depicted in Table 3, below.

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Table 3Beta-Galactosidase Specific Activity (nmol/min/mg)

	Virus	None	HSV	None	FHV
	Promoter	$\alpha 4$	$\alpha 4$	$\alpha 4$	$\alpha 4$
5	Cell	Vero	Vero	CRFK	CRFK
	Activity	5.1	31	10.3	28
	Virus	None	HSV	None	FHV
	Promoter	FHVtk	FHVtk	FHVtk	FHVtk
10	Cell	Vero	Vero	CRFK	CRFK
	Activity	not tested	not tested	1.2	2.8
	Virus	None	HSV	None	FHV
	Promoter	CMVIE	CMVIE	CMVIE	CMVIE
15	Cell	Vero	Vero	CRFK	CRFK
	Activity	32	106	35	100

Thus, all expression cassettes are believed to be active in FHV-infected cat CRFK cells, and the highest expression of β -galactosidase was from the CMV IE promoter. The highest activity reported in mock-transfected cells was 0.6 nmol/min/mg. Replacement of the beta-galactosidase coding sequence with, e.g., the FeLV envelope protein coding sequence, will produce an expression cassette that can be used as described above to construct an attenuated virus of the present invention that is suitable for use in vaccination against FHV and FeLV.

Other promoters that drive expression in FHV-infected cells can also be used in the construction of expression cassettes for use in the recombinant FHV's of the present invention. These include promoters derived from other herpes viruses, especially strongly-expressed promoters, as well as those derived from FHV, especially the major capsid protein gene promoter and the glycoprotein promoters (e.g., gB or gC homologues).

A number of plasmids containing the expression cassettes described in Table 3 inserted into the FHV TK gene of the present invention were constructed. These plasmids are referred to as insertion vectors, because when recombined with TK-positive FHV, the plasmids will insert the expression cassette-containing TK gene into

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the TK-positive FHV to yield a TK-minus FHV that contains the expression cassette. Thus, insertion vector pTC4 contains an expression cassette composed of the CMV IE promoter positioned to drive expression of beta-galactosidase.

5 Insertion plasmid pTC4 was transfected, together with infectious FHV genomic DNA, into CRFK cells using methods similar to those developed by Roizman et al. in the herpes simplex virus system (see Roizman et al., 1981, Cell 25:227-232; Roizman et al., 1981, Cell 24:555-565; Roizman et al., 1980, Cell 22:243-255; Roizman et al., 10 1982, Dev. Biol. Standardization 52:287-304; European Patent Publication 074,808; Roizman et al., 1985, Science 229:1208-1214) and by Lowe et al., 1987, Proc. Natl. Acad. Sci. 84:3896-3900, in the varicella zoster virus system.

15 Infectious FHV DNA can be generated by infecting subconfluent monolayers of CRFK cells with FHV at low multiplicity of infections. Cells are harvested when the cytopathic effect (CPE) has reached maximum. Cytoplasmic viral DNA is obtained by first removing cell nuclei by NP-40 extraction and treating the cytoplasmic fraction with 100 µg/ml proteinase K and 0.2% SDS (sodium dodecyl sulfate) for two 20 hours at 37°C. The viral DNA is purified by sodium iodide density ultracentrifugation. The DNA is then dialyzed and used directly in transfections to generate recombinants.

To generate virus recombinants, the plasmid containing the expression cassette inserted into the TK gene is cotransfected with 25 FHV DNA using the calcium phosphate precipitation method (Graham and vander Eb, 1973). 1 µg of plasmid DNA and 3 µg of FHV DNA are coprecipitated in 125 mM CaCl₂ and 1X Hepes buffered saline (HBS) at room temperature for 30 minutes. This precipitate is added to a 25 cm² subconfluent dish of CRFK cells with 5 ml of DMEM medium 30 supplemented with 10% fetal calf serum (FCS). After four hours, the cells are washed with DMEM (10% FCS) and incubated in 15% glycerol, 1X HBS for six minutes. The cells are washed and incubated in DMEM (10% FCS) until complete CPE is detected. The virus stock is harvested by freeze-thawing and sonication. Plaques are isolated by 35 incubation in Medium 199 supplemented with 0.5% agarose, 1% FCS, 100 µg/ml thymine arabinoside (araT). X-gal is added (300 µg/ml) after 48 hours if beta-galactosidase activity is to be detected. Blue plaques develop three days post infection and are picked, transferred

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to 1 ml of Medium 199 (1% FCS), sonicated and used to infect monolayers of CRFK cells. This process of plaque purification is repeated three times to generate a homogenous viral stock.

Progeny virus were harvested and plaqued on CRFK cells in the presence of 100 ug/ml araT, a thymidine analogue that selects for TK-minus virus. Plaques were stained for beta-galactosidase activity by including the chromogenic indicator X-gal (5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside) in the agar overlay, as described by Spaete *et al.*, 1987, *Proc. Natl. Acad. Sci.* 84:7213-7217.

Approximately five percent (5%) of the araT-resistant, TK-minus FHV plaques stained for beta-galactosidase expression developed the blue color indicative of the presence of beta-galactosidase activity on X-gal indicator plates. These viruses are plaque-purified as described above. These viruses express both the araT-resistant (TK-minus) and beta-galactosidase-positive phenotypes. The insertion of the CMV IE promoter/beta-galactosidase expression cassette within the TK gene of the FHV genome is confirmed by Southern analysis of FHV genomic DNA. This virus can be used as an attenuated FHV vaccine (and beta-galactosidase expression vector) for cats. Replacement of the beta-galactosidase gene with the envelope gene of FeLV subgroup A within a TK-based insertion plasmid will yield, via similar methods, a recombinant TK-minus FHV of the present invention that can drive expression of the FeLV envelope gene product in cats. Such a recombinant FHV can be used as a vaccine for FeLV and FHV.

Convenient methods other than TK-minus selection to select the desired recombinant virus are feasible as a result of work described in this application. As has been done with recombinant vaccinia virus vectors (Chakrabarti *et al.*, 1985, *Mol. Cell. Bio.* 5:3403-3409), a cassette capable of driving expression of beta-galactosidase can be co-inserted with another heterologous protein expression cassette into, for example, the TK gene, and these two genes can be together transferred into the viral genome. Recombinant viruses are then readily screened by virtue of their staining with the beta-galactosidase specific chromogenic reagent X-gal. Thus, for example, the CMV IE promoter/beta-galactosidase expression cassette can be inserted along with the FHV TK promoter/FeLV envelope gene product expression cassette into the FHV TK gene as described above, and recombinant viruses could be isolated by staining with X-gal.

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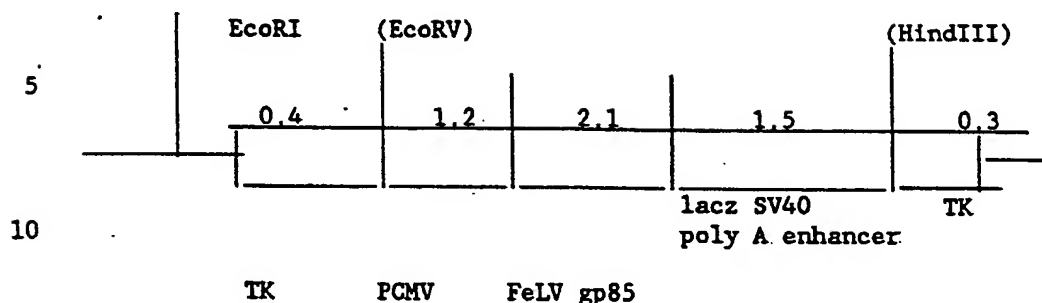
If a TK-positive phenotype is desired in a recombinant TK-minus virus in which the heterologous expression cassette is inserted within the TK gene, then a functional FHV TK gene can be inserted elsewhere in the genome. As current anti-herpes virus therapy acts
5 through a functional TK gene, it may be desirable to include a functional TK gene in the vaccine strain. This has not been done in the case of currently approved recombinant TK-minus pseudorabies virus vaccines (TechAmerica, Omni-Vac PRV). Attenuation by virtue of the TK-minus phenotype can be obtained by interrupting the FHV TK
10 gene, regardless of the site of integration of the expression cassette. In addition, the parental FHV virus can itself be attenuated through other means, independent of the TK gene. Conventionally produced attenuated FHV viruses are in current use as FHV vaccines.

15 The use of FHV as a vector for vaccination in cats is preferred to any other virus, including vaccinia virus. FHV replicates well in cats, and attenuated viruses are in current use in vaccination. Furthermore, the virus host-range is restricted to felines - - this virus is not presently known to infect other animals and humans and
20 thus does not pose the same public health concerns as vaccinia virus (which is considered a class 2 pathogen, because vaccination with the virus for smallpox immunization was ceased several years ago).

Recombinant virus construction: A bacterial plasmid containing a deletion in the identified FHV tk gene was constructed using
25 standard molecular cloning techniques. This plasmid and FHV strain UT88-1729 DNA were cotransfected into CRFK cells by using the calcium phosphate precipitation method described by Graham, F.L. and A.J. vander Eb., 1973, "A New Technique for the Assay of Infectivity of adenovirus 5 DNA", Virology 52: 446-467. Progeny virus was harvested
30 when full cytopathic effect was evident and recombinant FHV plaques were isolated in the presence of araT. The desired recombinant virus was identified by restriction endonuclease analysis.

Construction of recombinant tk-FHV:

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FeLV Envelope Insertion

The diagram above represents an FHV-gp85 recombinant virus (FHV114).
 15 The sequence from the EcoRV site to the HindIII site in the thymidine
 kinase gene has been deleted, attenuating the virus. An expression
 cassette including the FeLV gp85 gene has been inserted. The
 promoter in this cassette is from the CMV immediate early gene
 (Thomsen et al, PNAS 81:659-663)1984). The polyadenylation signal
 20 was isolated from pON1 [Spaete and Mocarski, J. Virol. 56:135-143
 (1985)]. CRFK cells infected with this virus synthesize FeLV gp85.

To obtain genetic and biochemical confirmation that the
 identified tk gene encodes FHV tk, we constructed a recombinant FHV
 in which the tk coding sequence had been modified to delete the
 25 nucleoside binding domain of the deduced tk protein.

The bacterial plasmid ptkΔEcoRV-HindIII (pGC113) contains the
 entire FHV tk gene and flanking regions (from the SalI site to the
 proximal BamHI site), but lacks coding sequences between the EcoRV
 and HindIII sites. A synthetic oligonucleotide polylinker was used
 30 to join these sites in the plasmid construction. The resulting
 protein is predicted to contain a novel serine residue inserted at
 the site of the glycine₁₁₇ to lysine₂₃₄ deletion.

This mutation was introduced into FHV by using calcium phosphate
 co-precipitation techniques to obtain homologous recombination
 35 between plasmid and herpesvirus genomic sequences. The plasmid
 ptkΔEcoRV-HindIII and FHV strain UT88-1729 genomic DNA were
 cotransferred into CRFK cells and progeny virus was harvested and
 plaqued onto CRFK cells in the presence of 100 μg/ml thymidine
 arabinoside (araT) to select for recombinant tk⁻ virus. Previous
 40 studies had shown this thymidine analogue to provide stringent
 selection against the replication of tk⁺ FHV, R.F. Schinazi, C.C.

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Williams, M.E.Fritz and A.J. Nahmias, in the Human Herpesviruses, Elsevier, New York, 1981, pp. 681-682, and we have used this selection method to isolate spontaneous tk⁻ FHV. AraT-resistant viruses were screened by restriction endonuclease analysis for the presence of the EcoRV-HindIII deletion. All araT-resistant viruses examined contained the expected deletion. One virus was further plaque purified and was designated FHV-113. As expected, the 6.6 kb EcoRI fragment containing the FHV tk gene is reduced in size by approximately 345 bp in FHV-113 (Fig. 1).

10 The araT-resistant phenotype of FHV-113 was shown to be attributable to a defect in tk by direct enzymatic assay of tk activity in extracts of infected cells. Results of these assays (Fig. 2) confirm the araT-resistant FHV-113 to be deficient in tk enzymatic activity. Thus, genetic and biochemical analysis supports the assignment based on the deduced amino acid sequence, and establishes that the identified gene encodes FHV tk.

Example 1 Construction of a tk deletion of FHV-1

The FHV113 virus was constructed as follows. pTK 3.8, described above, contains the 3.8 kb SalI/HindIII fragment containing the N-terminal coding sequences of the tk gene. A derivative of this plasmid, tk3.8ΔEcoRI was obtained by deletion of sequences between the EcoRI site in the TK promoter and in the Blue Script KS polylinker.

25 The plasmid pTK5.4, described earlier, contains the 5.4 kb HindII/EcoRI fragment containing the C-terminal coding region of the tk gene and the glycoprotein H gene. p5.4ΔBamHI was constructed by deletion of the sequences from the BamHI site just downstream from tk to the BamHI site in the Blue Script SK polylinker.

30 pGCIII was assembled by ligating the ApaI/EcoRI fragment from ptk3.8ΔEcoRI containing the region upstream from the tk gene, plus the HindIII/ApaI fragment from ptk5.4ΔBamHI, using a synthetic oligonucleotide to link the HindIII and EcoRI cleavage sites. The resulting sequence between the EcoRI and HindIII sites is GAATTGCGGGCCGCAAGCTT.

35 The insertion vector pGC113 was derived from pGCIII by inserting an EcoRI/EcoRV fragment containing the 5'-end of the tk gene (bases 321-740 in the above DNA sequence), between the EcoRI and HindIII sites of pGCIII, using a synthetic oligonucleotide to linker the

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EcoRV and HindIII sites. The resulting sequence between the EcoRV and HindIII sites was GGATCCAAGCTT, to regenerate the HindIII site and create a novel BamHI site.

The parent of the tk deletion virus was a highly virulent strain, UT88-1729 (obtained from Malcolm Martin, University of Tennessee Veterinary Teaching Hospital, Knoxville, Tennessee). Viral DNA was prepared from sodium dodecyl sulfate-proteinase K treated cytoplasmic nucleocapsids by the method of Walboomers and Scheggett (Virology, 74, 256-258, 1976), and described above. Using the transfection protocol described above, FHV DNA plus pGC113 DNA was transfected into CRFK cells. A thymidine kinase negative plaque was isolated by thymidine arabinoside selection.

The resulting virus, designated FHV-113, contains a tk deletion. Its DNA, analyzed by restriction enzyme EcoRI, is shown in Fig. 1 and its tk⁻ phenotype is demonstrated in Fig. 2.

Example 2 Construction of a FHV expressing FeLV gp85

The plasmid pON1 contains an E. coli beta galactosidase gene and SV40 polyadenylation signal (Spaete, et al., J. Virol., 56, 135-143, 1985) and was obtained from Ed Mocarski, Stanford University. pON-CMVIE, described earlier, contains the PstI/SocII fragment of the human cytomegalovirus major immediate early promoter in pON1.

Sequences from the feline leukemia virus A subgroup (strain Glasgow-1 genome are cloned in the plasmid pFGA-5, obtained from Dr. James Neil, University of Glasgow. The construction of this clone, its restriction enzyme cleavage map, and relevant DNA sequence is described in Stewart, et al., J. Virol., 58, 825-834, 1986. The env gene was isolated as a PstI/PstI fragment, inserted into pUC19 (Pharmacia, Piscataway, N.J.) to obtain convenient flanking restriction sites (XbaI on the 5'-end, SphI, which can be made blunt with T4 DNA polymerase, on the 3'-end). Plasmid pCMVIE-FeLVenv was made by replacing the beta-galactosidase gene of pON-IECMV with FeLV env.

The CMV promoter - FeLV env expression cassette was removed from pCMVIE-FeLVenv and inserted into the tk insertion vector pGC113 (Example 1) to give plasmid pGC114. This plasmid contains the env transcription unit in the same orientation as the FHV tk gene. This plasmid was co-transfected with FHV UT88-1729 DNA into CRFK cells, and araT resistant plaques selected, as described above. The

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resulting virus was called FHV-114. FHV 114 directs expression of FeLV gp85 in infected CRFK cells, as determined by Western blotting or immunoprecipitation with various anti-FeLV monoclonal or polyclonal antisera (obtained from Dr. Niels Pedersen, U. California, Davis). Intranasal administration of this virus to cats was found to induce antibodies to FeLV.

Example 3 Vaccination of cats with recombinant FHV vaccine

FHVA113 as unformulated material, i.e., medium from cells infected with FHVA113, was administered to cats by intranasal inoculation. Unlike the parent virus UT88-1729, which caused severe or even/lethal disease, the FHVA113-inoculated cats did not show signs of illness. These cats developed antibodies that neutralized FHV. When these cats were challenged with virulent FHV strains, they did not develop respiratory symptoms as did unvaccinated cats. This demonstrates that thymidine kinase negative FHVs are avirulent and raise a protective immune response, neither of which was known before this invention.

Those skilled in the art will recognize, in light of the present disclosure, that the methods of the claimed invention can be carried out in a variety of ways. The exemplifications of the invention described above merely illustrate the invention and in no way limit the scope of the accompanying claims. Other modifications of the above-described embodiments of the invention that are obvious to those skilled in the art are intended to be within the scope of the following claims. Particularly well known are methods for formulating and administering vaccines comprising live attenuated viruses such as those of the instant invention.

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CLAIMS

1. A method for isolating thymidine kinase-encoding DNA from a herpes virus that comprises:

(a) mixing thymidine kinase-encoding DNA of a herpes virus with
5 a mixture of primers, said mixture of primers containing primers that
encode, in all variations possible due to the degeneracy of the
genetic code, all variations of a first sequence of conserved amino
acids of herpes virus thymidine kinase proteins, and primers that
10 encode, in all variations possible due to the degeneracy of the
genetic code, sequences complementary to sequences that encode all
variations of a second sequence of conserved amino acids of herpes
thymidine kinase proteins;

(b) amplifying the thymidine kinase-encoding DNA by the
polymerase chain reaction; and

15 (c) isolating the amplified DNA.

2. The method of Claim 1 that further comprises:

(a) hybridizing said amplified DNA to a herpes virus genomic
library that comprises recombinant DNA vectors that comprise herpes
20 virus genomic DNA; and

(b) isolating said vectors that hybridize.

3. The method of Claim 1, wherein said sequences of conserved amino
acids are selected from the group consisting of DGPHG and DGAYG, GKTT
25 and GKST, DRHP and DRHA, RPGE and RAGE, and DTLF, wherein A is
alanine, D is aspartic acid, E is glutamic acid, F is phenylalanine,
G is glycine, H is histidine, K is lysine, L is leucine, P is
proline, R is arginine, S is serine, T is threonine, and Y is
tyrosine.

30

4. The method of Claim 1, wherein said variations of a first
sequence of conserved amino acids are DGPHG and DGAYG and said
variations of a second sequence of conserved amino acids are DRHP and
DRHA, and wherein A is alanine, D is aspartic acid, G is glycine, H
35 is histidine, P is proline, R is arginine, and Y is tyrosine.

5. A herpes virus thymidine kinase-encoding DNA isolated by the
method of Claim 1.

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6. A recombinant DNA molecule comprising a feline herpes virus thymidine kinase-encoding DNA.

- 5 7. The recombinant DNA molecule of claim 6 wherein the thymidine kinase-encoding DNA comprises the following DNA sequence:

5'-AT GGCGAGTGGG
ACCATCCCCG TTCAGAAATGA AGAGATTATT AAATCACAGG TGAATACTGT
10 CCGCATTAC ATAGATGGTG CCTATGGAAT AGGTAAGAGT TTAACGGCGA
AGTACCTGGT CAGAGCGGAT GAAAATCGAC CGGGATATAC TTACTACTTC
CCAGAACCAA TGCTATACTG GCGTAGTCTC TTTGAAACTG ATGTTGTCGG
TGGTATCTAT GCGTCCAGG ACCGGAAACG ACGTGGTGAA TTATCAGCTG
AAGATGCTGC CTATATCACC GCCCACTATC AAGCAAGATT TGCCGCACCA
15 TACCTTCTTT TACATTCCAG ACTATCCACA ATAACAGGAT ATCAGAAACT
TGTATGTGAG GAACACCCCG ACGTGACCCT AATCATAGAT AGACACCCTC
TCGCCTCTCT GGTCTGTTTC CCACTCGCAA GATATTTTGT GGGTGATATG
ACTCTTGGGT CTGTACTTAG TCTAATGGCA ACACTTCCAC GAGAACCTCC
TGGTGAAAT CTAGTTGTAA CAACCTGAA TATCGAGGAA CATTGGAAGC
20 GTCTCAGGGG ACGCTCAAGA ACCGGAGAAC AGATAGACAT GAAGCTAATT
CACGCACTAC GCAATGTATA TATGATGTTG GTACATACTA AGAAATTTT
AACAAAAAAT ACTAGTTGGC GTGATGGGTG GGGGAAGCTT AAAATTTTCT
CCCACTATGA ACGGAATAGG CTCGTGAAA CTACAATAGT TTCCGATTGG
ACGGAGTCAG ATTTATGTGA CACATTATTC AGTGTTTTCA AAGCCCGGGA
25 GCTCTCCGAC CAAAATGGAG ATCTACTTGA CATGCATGCA TGGGTCCTCG
ATGGACTTAT GGAAACCCTC CAAAATTAC AGATCTTTAC TTAAATCTG
GAAGGAACCC CTGATGAATG TGCCGCCGCC TTGGGAGCAC TGAGACAAGA
TATGGATATG ACATTTATAG CCGCATGTGA TATGCACCGT ATAAGTGAAG
CCTTGACGAT ATACCATTA-3'

30

8. The recombinant DNA molecule of Claim 7 wherein the DNA sequence is:

5'-GTATAACCAC AGATCTGTAT GTTCAACCTC ACGACGTTGA TGTCTTACTA
GTGTATCCAT ATTTGAAAA CGACACGTTT TCAGCTCAAT TAGAAAACAT
35 ATACCACCCC CTTCTCCCTC AAATTGTATA GTACATACAC AATCAGTCGG
CGACGACCCA AGTTAACCTC ACATGCTAGG TACACGCCCT TAGCCTTTT
AAGAGACTCT GCGGATACAG AGCCGCCCAA TAAACACTCG AGTCGGTCGG
TATATACTCC ACTCGCAGAG GTCGAGGATA TATCGCGCTT GAGGACAGCA

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TAAAAGCGAT TGTGGNATCG AATTCAGCC CGGAGCCTCA ATCCGACACT
 GCGTCGTTGT TCACGTTTCA TCATACACAG ATCAGACGAT GGCGAGTGGG
 ACCATCCCCG TTCAGAATGA AGAGATTATT AAATCACAGG TGAATACTGT
 CCGCATTAC ATAGATGGTG CCTATGGAAT AGGTAAGAGT TTAACGGCGA
 5 AGTACCTGGT CAGAGCGGAT GAAAATCGAC CGGGATATAC TTACTACTTC
 CCAGAACCAA TGCTATACTG GCGTAGTCTC TTTGAAACTG ATGTTGTCCG
 TGGTATCTAT GCCGTCCAGG ACCGGAAACG ACGTGGTGAA TTATCAGCTG
 AAGATGCTGC CTATATCACC GCCCACTATC AAGCAAGATT TGCCGCACCA
 TACCTTCTTT TACATTCCAG ACTATCCACA ATAACAGGAT ATCAGAAAGT
 10 TGTATGTGAG GAACACCCCG ACGTGACCCT AATCATAGAT AGACACCGTC
 TCGCCTCTCT GGTCTGTTTC CCACTCGCAA GATATTTTGT GGGTGATATG
 ACTCTTGGGT CTGTACTTAG TCTAATGGCA ACACTTCCAC GAGAAGCTCC
 TGGTGGAAT CTAGTTGTAA CAACCTTGAA TATCGAGGAA CATTGGAAGC
 GTCTCAGGGG ACGCTCAAGA ACCGGAGAAC AGATAGACAT GAAGCTAATT
 15 CACGCACTAC GCAATGTATA TATGATGTTG GTACATACTA AGAAATTTT
 AACAAAAAAT ACTAGTTGGC GTGATGGGTG GGGGAAGCTT AAAATTTTCT
 CCCACTATGA ACGGAATAGG CTCGTGAAA CTACAATAGT TTCCGATTCC
 ACGGAGTCAG ATTTATGTGA CACATTATTC AGTGTTTTCA AAGCCCCGGA
 GCTCTCCGAC CAAAATGGAG ATCTACTTGA CATGCATGCA TGGGTCTCTG
 20 ATGGACTTAT GGAAACCTC CAAAATTTAC AGATCTTTAC TTAAATCTG
 GAAGGAACCC CTGATGAATG TGCCGCCGCC TTGGGAGCAC TGAGACAAGA
 TATGGATATG ACATTIATAG CCGCATGTGA TATGCACCGT ATAAGTGAAG
 CCTTGACGAT ATACCATTAA ACATTAGTGG TGTTCCTAT TACCCCCCTG
 TGGTGAATGT GTGGAGGTCA GGGGATAATT GTATAATGAC CATCGTTTCA
 25 TGAATAAAAT AACCGTGTGT GATGTGGATG TATTCATTAA TTGAATTTCT
 CTTCCGGTTT TAGATCTTAA TAAGCGTAAA ACTGGTGTTC TAAATCCAAG
 AGCCGGGTTT TTTGGAGGTT GGTACATCA TCGCCACAGC CCGTGGATTC
 AAGCAATCTT ATGATGTGTT TGATAATATA CCTATCGATA TTCCTGATCA
 TTGTATCGAG GATGTTGACT GGTTACCGAT GATGGATAGA CCTGATGAGG
 30 TGGCTGG-3

9. A feline herpes virus thymidine kinase-encoding DNA sequence
 that encodes the amino acid residue sequence, depicted from the amino
 to carboxy terminus:

35 MASGTIPVQN EEIISQVNT VRIYIDGAYG IGKSLTAKYL VRADENRPGY
 TYYFPEPMLY WRSLFETDVV GGIYAVQDRK RRGELSAEDA AYITAHYQAR
 FAAPYLLHS RLSTITGYQK VVCEEHPDVT LIIDRHPLAS LVCFLPARYF
 VGDMTLGSLV SIMATLPREP PGGNLVVTTL NIEEHLKRLR GRSRTGEQID

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MKLIHALRNV YMMLVHTKKF LTKNTSWRDG WGKLIKFSHY ERNRLVETTI
 VSDSTESDLC DTLFSVFKAR ELSDQNGDLL DMHAWVLDGL METLQNLQIF
 TLNLEGTPDE CAAALGALRQ DMDMTFIAAC DMHRISEALT IYH

wherein, Alanine is A, Arginine is R, Asparagine is N, Aspartic acid
 5 is D, Cysteine is C, Glutamine is Q, Glutamic acid is E, Glycine is
 G, Histidine is H, Isoleucine is I, Leucine is L, Lysine is K,
 Methionine is M, Phenylalanine is F, Proline is P, Serine is S,
 Threonine is T, Tryptophan is W, Tyrosine is Y, and Valine is V.

10 10. A method for constructing recombinant thymidine kinase-negative
 feline herpes viruses that comprises:

(a) constructing a recombinant DNA vector that encodes a non-
 functional feline herpes virus thymidine kinase gene;

(b) transfecting a feline herpes virus-permissive host cell
 15 with a mixture of thymidine kinase-positive feline herpes virus and
 said vector that encodes a non-functional thymidine kinase gene;

(c) isolating the progeny virus;

(d) infecting a feline herpes virus-permissive host cell with
 said progeny virus to produce thymidine kinase-negative virus; and

20 (e) isolating said thymidine kinase-negative virus.

11. The method of Claim 10, wherein said amplifying step comprises
 culturing said infected host cells in the presence of araT.

25 12. The method of Claim 10, wherein said non-functional thymidine
 kinase comprises a portion of the DNA sequence:

5'-GTATAACCAC AGATCTGTAT GTTCAACCTC ACGACGTGA TGTCTTACTA
 GTGTATCCAT ATTTGAAAA CGACACGTTT TCAGCTCAAT TAGAAAACAT
 ATACCACCCC CTTCTCCCTC AAATTGTATA GTACATACAC AATCAGTCGG
 30 CGACGACCCA AGTTAACCTC ACATGCTAGG TACACGCCCT TAGCCTTTTT
 AAGAGACTCT GCGGATACAG AGCCGCCCAA TAAACACTCG AGTCGGTCCG
 TATATACTCC ACTCGCAGAG GTCGAGGATA TATCGCGCTT GAGGACAGCA
 TAAAAGCGAT TGTGCGATCG AATTCCAGCC CGGAGCCTCA ATCCGACACT
 GCGTCGTTGT TCACGTTTCA TCATACACAG ATCAGACGAT GCGGAGTGG
 35 ACCATCCCCG TTCAGAATGA AGAGATTATT AAATCACAGG TGAATACTGT
 CCGCATTTAC ATAGATGGTG CCTATGGAAT AGGTAAGAGT TTAACGGCGA
 AGTACCTGGT CAGAGCGGAT GAAAATCGAC CGGGATATAC TTACTACTTC
 CCAGAACCAA TGCTATACTG GCGTAGTCTC TTTGAAACTG ATGTTGTCCG

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TGGTATCTAT GCCGTCCAGG ACCGGAAACG ACGTGGTGAA TTATCAGCTG
 AAGATGCTGC CTATATCACC GCCCACTATC AAGCAAGATT TGCCGCACCA
 TACCTTCTTT TACATTCCAG ACTATCCACA ATAACAGGAT ATCAGAAAAGT
 TGTATGTGAG GAACACCCCG ACGTGACCCT AATCATAGAT AGACACCCTC
 5 TCGCCTCTCT GGTCTGTTT CCACTCGCAA GATATTTTGT GGGTGATATG
 ACTCTTGGGT CTGTACTTAG TCTAATGGCA ACACTTCGAC GAGAACCCTC
 TGGTGGAAAT CTAGTTGTAA CAACCTTGAA TATCGAGGAA CATTTGAAGC
 GTCTCAGGGG ACGCTCAAGA ACCGGAGAAC AGATAGACAT GAAGCTAATT
 CACGCACTAC GCAATGTATA TATGATGTTG GTACATACTA AGAAATTTTT
 10 AACAAAAAAT ACTAGTTGGC GTGATGGGTG GGGGAAGCTT AAAATTTTCT
 CCCACTATGA ACGGAATAGG CTCGTGGAAG CTACAATAGT TTCCGATTCC
 ACGGAGTCAG ATTTATGTGA CACATTATTC AGTGTTTTCA AAGCCCGGGA
 GCTCTCCGAC CAAAATGGAG ATCTACTTGA CATGCATGCA TGGGTCCTCG
 ATGGACTTAT GGAAACCCTC CAAAATTAC AGATCTTIAC TTAAATCTG
 15 GAAGGAACCC CTGATGAATG TGCCGCCGCC TTGGGAGCAC TGAGACAAGA
 TATGGATATG ACATTTATAG CCGCATGTGA TATGCACCGT ATAAGTGAAG
 CCTTGACGAT ATACCATTAA ACATTAGTGG TGTTCCTAT TACCCCCCTG
 TGGTGAATGT GTGGAGGTCA GGGGATAATT GTATAATGAC GATCGTTTCA
 TGAATAAAAT AACCGTGTGT GATGTGGATG TATTCATTAA TTGAATTTCT
 20 CTTCGGGTTT TAGATCTTTA TAAGCGTAAA ACTGGTGTTT TAAATCCAAG
 AGCCGGGTTT TTTGGAGGTT GGTACATCA TCGCCACAGC CCGTGGATTG
 AAGCAATCTT ATGATGTGTT TGATAATATA CCTATCGATA TTCCTGATCA
 TTGTATCGAG GATGTTGACT GGTACCGAT GATGGATAGA CCTGATGAGG
 TGGCTGG-3

25

13. The method of Claim 10, wherein said non-functional thymidine kinase gene is a segment of colinear feline herpes virus genomic DNA from which DNA necessary for the expression of thymidine kinase has been deleted.

30

14. The method of Claim 13, wherein said DNA necessary for the expression of thymidine kinase is the thymidine kinase gene.

35

15. A thymidine kinase-negative feline herpes virus.

16. A recombinant thymidine kinase-negative feline herpes virus according to Claim 15.

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17. A recombinant thymidine kinase-negative feline herpes virus according to Claim 16 comprising a non-functional thymidine-kinase gene comprising a portion of the DNA sequence of Claim 12.
- 5 18. A vaccine comprising the thymidine kinase-negative feline herpes virus of claim 15.
19. A vaccine comprising the thymidine kinase-negative feline herpes virus of claim 16.
- 10 20. A vaccine comprising the thymidine kinase-negative feline herpes virus of claim 17.
21. A recombinant thymidine kinase-negative feline herpes virus
15 produced by the method of Claim 10.
22. The recombinant feline herpes virus of Claim 16 that further comprises an expression cassette that comprises a promoter that can drive expression of a gene product in feline herpes virus-infected
20 cells and a coding sequence positioned for expression from said promoter.
23. The recombinant feline herpes virus of Claim 22, wherein said
25 promoter is a herpes virus promoter.
24. The recombinant feline herpes virus of Claim 23, wherein said promoter is selected from the group consisting of the herpes simplex alpha-4, cytomegalovirus immediate early, and feline herpes virus thymidine kinase promoters.
- 30 25. The recombinant feline herpes virus of Claim 24, wherein said promoter is the cytomegalovirus immediate early promoter.
26. The recombinant feline herpes virus of Claim 22, wherein said
35 coding sequence encodes a viral gene product.
27. The recombinant feline herpes virus of Claim 26 wherein said gene product is selected from the group consisting of feline leukemia

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virus, feline infectious peritonitis (FIP) virus, calicivirus, rabies virus, feline immunodeficiency virus (FIV), feline parvovirus (panleukopenia virus), and feline Chlamydia;

5 28. The recombinant feline herpes virus of Claim 26, wherein said viral gene product is a gene product of feline leukemia virus.

29. The recombinant feline herpes virus of Claim 28, wherein said gene product is selected from the envelope, gag, and pol gene
10 products.

30. The recombinant feline herpes virus of Claim 29 wherein said gene product is a secreted envelope gene product.

15 31. A vaccine comprising the recombinant feline herpes virus of claim 22.

32. A vaccine comprising the recombinant feline herpes virus of claim 30.

20

33. A recombinant DNA molecule that comprises the thymidine kinase gene promoter of feline herpes virus.

25 34. The recombinant DNA molecule of Claim 33 that comprises the DNA sequence:

5'-CAATAAACACTCGAGTCGGTCGGTATATACTCCACTCGCAGAGGTCGAGGATATAT.

35. The recombinant DNA molecule of Claim 34 that comprises the DNA sequence:

30 5'-GTATAACCAC AGATCTGTAT GTTCAACCTC ACGACGTTGA TGTCTTACTA
GTGTATCCAT ATTTTGAAAA CGACACGTTT TCAGCTCAAT TAGAAAACAT
ATACCACCCC CTTCTCCCTC AAATTGTATA GTACATACAC AATCAGTCGG
CGACGACCCA AGTTAACCTC ACATGCTAGG TACACGCCCT TAGCCTTTTT
AAGAGACTCT GCGGATACAG AGCCGCCCAA TAAAGACTCG AGTCGGTCGG
35 TATATACTCC ACTCGCAGAG GTCGAGGATA TATCGCGCTT GAGGACAGCA
TAAAAGCGAT TGTGGNATCG AATTCCAGCC CGGAGCCTCA ATCCGACACT
GCGTCGTTGT TCACGTTTCA TCATACACAG TCAGACG-3'.

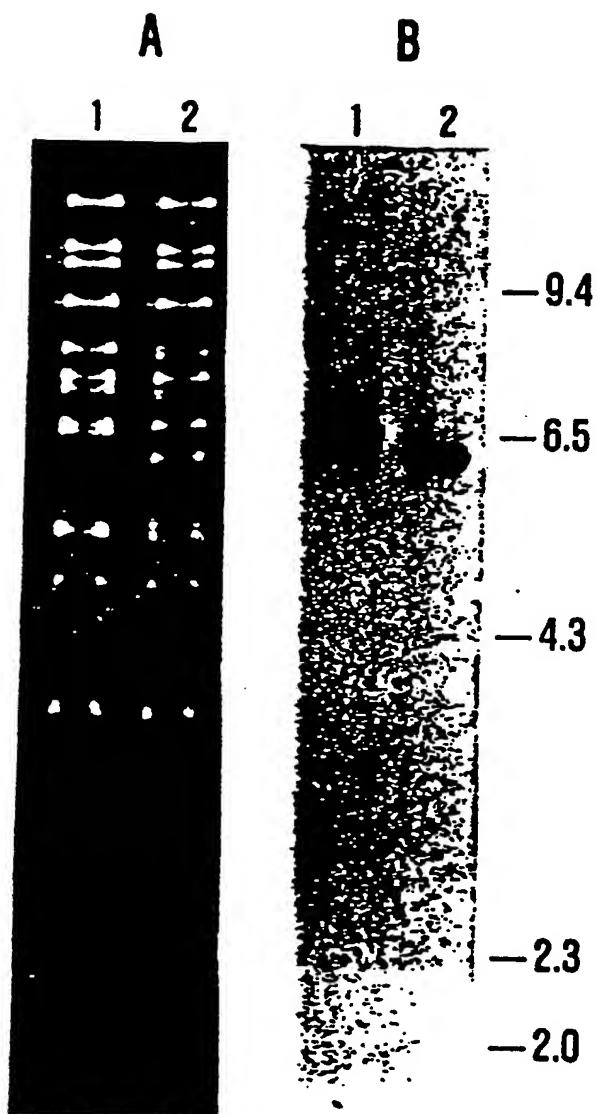
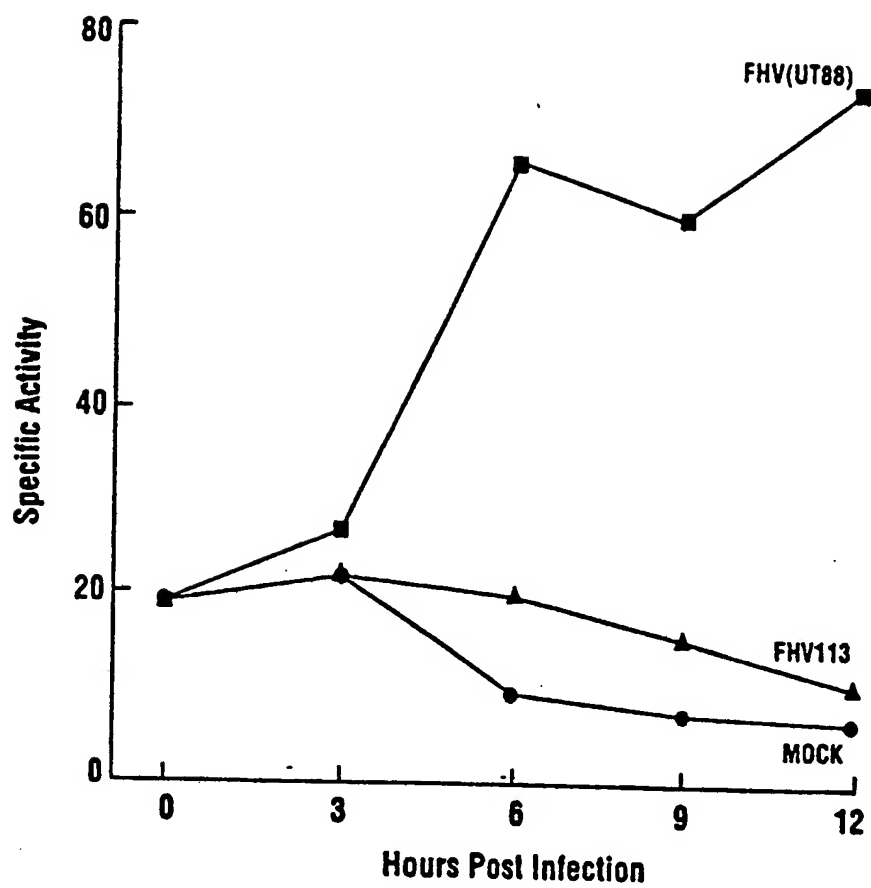
FIGURE 1

FIGURE 2.



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03289

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 12 N 15/10, C 12 N 15/38, C 12 N 15/86, A 61 K 39/245, IPC ⁵ : 39/12, 39/21, 39/285		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 12 N, A 61 K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP, A, 0216564 (CETUS CORP.) 1 April 1987,- see example 5; claims --	10-32
Y	WO, A, 87/04463 (SYNTRO CORPORATION) 30 July 1987, see example 16; claims --	10-32
X	EP, A, 0251534 (MERCK & CO.) 7 January 1988, see page 6, lines 20-24 --	5
A	Proc. Natl. Acad. Sci, USA, vol. 81, September 1984, (US) M.F. Shih et al.: "Expression of hepatitis B virus S gene by herpes simplex virus type 1 vectors carrying alpha- and beta-regulated gene chimeras", pages 5867-5870 see page 5867 --	
A	EP, A, 0201184 (CETUS CORP.) 17 December 1986, see page 5, column 7, line 48 - column 8, line 19 (cited in the application) --	1-4
<p>* Special categories of cited documents: ¹⁴</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
24th November 1989	29.12.89	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	F.M. VRIJDAG	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, vol. 105, no. 23, 8 December 1986, (Columbus, Ohio, US), P.A. Rota et al.: "Physical characteri- zation of the genome of feline herpesvirus-1", see page 136, abstract 203931p & Virology, 1986, 154(1), 168-79	
P,X	Journal of Virology, vol. 63, no. 8, August 1989, American Soc. for Microbiology (US) J.H.Nunberg et al.: "Identification of the thymidine kinase gene of feline herpesvirus: use of degenerate oligo- nucleotides in the polymerase chain reaction to isolate herpesvirus gene homologs", pages 3240-3249, see page 3240	1-32

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8903289

SA 30853

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 19/12/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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		JP-A- 62151186	06-07-87
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		EP-A- 0256092	24-02-88
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